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X-ray crystallographic identification of a protein-binding site for both all-*trans*- and 9-*cis*-retinoic acid

(epididymis/retinoic acid-binding protein)

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ABSTRACT **The elucidation of how a protein-binding site might specifically recognize both the all-trans and 9-cis isomers of retinoic acid is of particular interest because of the recently discovered binding specificities of the nuclear receptors for retinoic acid. Two families of nuclear receptors for retinoic acid have been described, which are designated RAR (for retinoic acid receptor) and RXR (for retinoid-X receptor). The RXR family of receptors is specific for 9-*cis*-retinoic acid, whereas the RAR-type receptor is activated by either 9-*cis*- or all-*trans*-retinoic acid. During the x-ray structure determination of a secreted epididymal retinoic acid-binding protein, with and without retinoic acid, we observed an electron density for the bound all-*trans*-retinoic acid that indicates the protein-bound all-trans form of the vitamin/hormone adopts a horseshoe-like conformation that resembles the structure of the 9-cis isomer of the ligand. We detail here the experiments that indicate the electron density is indeed due to all-*trans*-retinoic acid and that protein can also bind the 9-cis isomer. This observation and the fact that the same protein also binds the synthetic retinoid (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]-benzoic acid (TTNPB), a retinoic acid analog that activates RAR but does not activate RXR, suggest that the mechanism by which this protein recognizes both 9-*cis*- and all-*trans*-retinoic acids may be analogous to the mechanism used by RAR. Three crystallographic structures of retinol-binding proteins have been described. In each of these structures the retinol binds with the isoprene tail fully extended. This report represents an x-ray crystallographic description of a protein-bound retinoid conformer that adopts a nonextended conformation, and we believe this observation is relevant to the ligand specificities described for the retinoic acid receptors.**

Retinoic acid is a modulator of gene expression for which two families of nuclear receptors have been described. One family [retinoic acid receptor (RAR) (1, 2)] binds both all-*trans*- and 9-*cis*-retinoic acid, whereas the second family [retinoid-X receptor (RXR) (3)] appears specific for the 9-cis isomer (4, 5). Details of retinoid recognition by proteins are available from the structures of three retinol-binding proteins, the serum retinol-binding protein (6, 7) and the cellular retinol-binding proteins types I and II (8, 9). In all three of these structures the all-*trans*-retinol is bound with the isoprene tail fully extended. These proteins do not bind 9-*cis*-retinol (10). Exactly how a single binding site for retinoic acid can accommodate both the 9-cis and all-trans isomers, yet still maintain a high degree of specificity, is not known. In the course of the x-ray structural determination of a retinoic acid-binding protein from rat epididymis (E-RABP) in our laboratory, we observed for crystals of the binding protein complexed with retinoic acid an electron density in the putative binding site that was inconsistent with an extended retinoid but was consistent with either 9-*cis*-retinoic acid or an all-*trans*-retinoic acid molecule rotated about the C8—C9 bond, which then resembles 9-*cis*-retinoic acid (Fig. 1*A*). This observation suggested that this binding site might accommodate either isomer. Only all-*trans*-retinoic acid had been examined (12, 13). But both all-*trans*- and 9-*cis*-retinoic acid are found in epididymal extracts (14). Such an ability to bind both 9-*cis*- and all-*trans*-retinoic acid would make the binding protein from rat epididymis a possible model for the retinoid-recognition site of RAR or provide an interesting contrast once that structure is solved. Here we report that E-RABP does indeed bind all-*trans*-retinoic acid in the rotated configuration and can also bind the 9-cis isomer. In addition, the retinoic acid analog (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]-benzoic acid (TTNPB, Fig. 1*B*), an activator of retinoic acid receptor α (3), also binds well to the epididymal protein, extending the possible similarities in retinoid-recognition sites between these two proteins.†

MATERIALS AND METHODS

Protein Purification and Ligand-Binding Studies. Retinoic acid-binding protein was purified as described (13). The protein is expressed in two forms, B and C, which differ at the amino-terminal end: the C form has an additional three amino acids. Results are reported for the C form; experiments with the B form gave equivalent results. The 9-cis isomer of retinoic acid was prepared by oxidative esterification of 9-*cis*-retinal (15). All-*trans*- and 13-*cis*-retinoic acid were purchased from Sigma. TTNPB was from M. Dawson (SRI International, Menlo Park, CA). Stock solutions of 1:1 retinoic acid isomers and TTNPB were prepared in dimethyl sulfoxide. The protein was incubated in Amicon Centricon-10 tubes with the retinoids in a 300-μl vol. Final incubation conditions were 1% dimethyl sulfoxide/10 μM retinoic acid-binding protein/20 μM retinoid for each retinoid added. The tubes were flushed with N_2 and incubated at 21°C for 1 hr and then incubated overnight at 4°C. To confirm that no significant changes in isomer composition had occurred, a 100-μl vol was withdrawn and extracted. The remaining 200 μl was diluted to 2.0 ml with phosphate-buffered saline and subsequently concentrated to 100 μl by centrifugation. The dilution and concentration was then repeated twice to remove unbound ligand. Control experiments with lysozyme confirmed that the retinoic acid that remained in the retentate with E-RABP was specifically bound. Ligand was extracted from

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Abbreviations: RAR, retinoic acid receptor; RXR, retinoid-X receptor; TTNPB, (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]-benzoic acid; E-RABP, epididymal retinoic acid-binding protein.

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†The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (reference 1EPB).

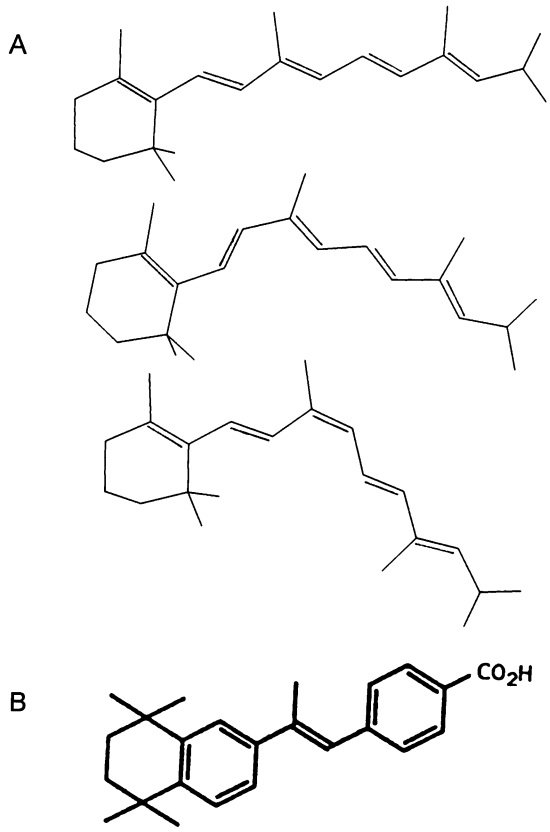

FIG. 1. (*A*) All-*trans*-retinoic acid from the coordinates of Stam (11), a conformer of all-*trans*-retinoic acid that resembles 9-*cis*-retinoic acid and 9-*cis*-retinoic acid. The all-trans conformer was produced by rotation about the C8—C9 single bond. A rotation about the C6—C7 single bond is also required to put the β-ionone ring in the same relative position. (*B*) Structure of TTNPB.

the protein with 2.0 ml of hexane after the addition of 2 vol of 0.05 M HCl/ethanol and 2 vol of 5 M NaCl to the protein samples. Analysis by adsorption HPLC was done as described (14).

Crystallization. Crystals of the holo-retinoic acid-binding protein were prepared from the purified B form of the protein to which a 20-fold excess of retinoic acid (dissolved in dimethyl sulfoxide) was added in 0.1% lauryldimethylamine oxide/10 mM Tris·HCl, pH 8.0. Excess retinoic acid was removed by passage over a C_{18} Sephadex column (13), although this step was not necessary to produce crystals with the reconstituted protein. Crystals grew in hanging-drop vapor-diffusion experiments from ammonium sulfate concentrations of 31% saturated ammonium sulfate/10 mM Tris·HCl, pH 8.0/10–12% (wt/vol) glycerol/2.5 mM EDTA at room temperature. These conditions resemble those for the apoprotein crystallizations, except that 40% saturated ammonium sulfate is used for apoprotein crystal growth. The crystals grew (with or without seeding with apo-crystals) as flat, thin, rectangular plates, a morphology quite distinct from that of the apo-crystals. However, the crystals belong to the same space group ($P2_1$) and are reasonably isomorphous with the apo-crystals: a = 39.8, b = 58.9, c = 66.6, β = 109.44° (holo) vs. a = 39.3, b = 58.7, c = 66.3, β = 109.35° (apo). There are two molecules of E-RABP per asymmetric unit.

X-Ray Data Collection and Map Calculation. X-ray data were taken with a pair of San Diego Multiwire area detectors mounted on a Rigaku RU-200 rotating anode. R_{merge} for the 94% complete data set was 6.88% for 53,778 reflections (14,116 unique). ($R_{merge} = \Sigma|I - \{I\}|/\Sigma\{I\}$, where I = intensity measurement for a given reflection and $\{I\}$ is the mean intensity for multiple measurements of the reflection.) The difference Fourier calculation was done with partial-structure phases (863 nonhydrogen atoms per monomer of a possible 1282 total) combined with the multiple isomorphous replacement phases (figure of merit after phase combination = 0.68) and ($F_{holo} - F_{apo}$) amplitudes. The map is contoured at two times the SD.

Structure Solution. The structure of the apo-retinoic acid-binding protein was solved by the method of multiple isomorphous replacement to 2.1 Å and will be reported elsewhere (16). The coordinates for the apoprotein with those water molecules not in the binding cavity were then used as the starting model for the holo-protein, for which we had diffraction data to 2.2 Å. The retinoic acid molecules were added, and the side chains of Met-39 in the two crystallographically independent protein molecules were positioned according to the electron density in the difference Fourier calculation. The structure was refined with the simulated annealing protocol of XPLOR 2.1 (17). The residual error (R) value for the current model is 18.2% for 94% of the data (those reflections with intensities better than two times the SD) between 6- and 2.2-Å resolution. The low-resolution data were included in the calculation of the $2F_o - F_c$ maps (F_o, observed structure factor amplitudes; F_c, calculated structure factor amplitudes). The calculation of omit maps, also including the low-resolution data, confirmed the placement of the retinoic acid molecules. The electron density for the retinoic acid of the protein molecule designated "A" was not as clear as that for "B" in either the original difference Fourier calculations or the $2F_o - F_c$ maps. The rms deviations from ideality for bond lengths and angles are 0.017 Å and 3.37°, respectively, for the current model. Phasing, phase combination, and map calculation before the use of XPLOR for refinement were done with the PHASES package (W. Furey, Veterans Administration Medical Center and University of Pittsburgh). For map interpretation O version 5.6 was used (18).

Extraction of Retinoic Acid from the Crystal. The crystals that were extracted had been in the hanging-drop experiments at room temperature for over 18 mo. Crystals were removed from the drops and washed with a large excess of freshly prepared "mother liquor" to remove any retinoic acid, or retinoic acid degradation products, which may have been free in the drops. The mother liquor was then removed, and the crystals were dissolved in water. All manipulations with the crystal, until it was dissolved in water, were done under a light microscope. The protein solution was extracted, as described above, for the solution studies, and the retinoic isomers were analyzed by HPLC (14).

RESULTS

To determine the x-ray crystallographic structure of E-RABP, both with and without ligand, crystals of holo-retinoic acid-binding protein were grown from reconstituted retinoic acid-E-RABP as described above. The crystallization of the apo form has been reported (13). The structure of the bound ligand was determined by standard difference Fourier methods: the structure of the apoprotein is "subtracted," so that only the additional electron density of the ligand, as well as that due to any conformational changes induced by the addition of ligand, is visible in the electron density map. The β-ionone ring of the retinoic acid was clearly visible in the difference Fourier calculated with the multiple isomorphous replacement phases and amplitudes derived from the holoprotein x-ray data. The ligand-binding site of E-RABP was found deep within the β-barrel structure of the protein. During the structural determination of the apo form of E-RABP, the multiple isomorphous replacement phases were improved by phase combination with those calculated from partial-structure information (863 of 1282 atoms per monomer). In the difference Fourier value calcu-

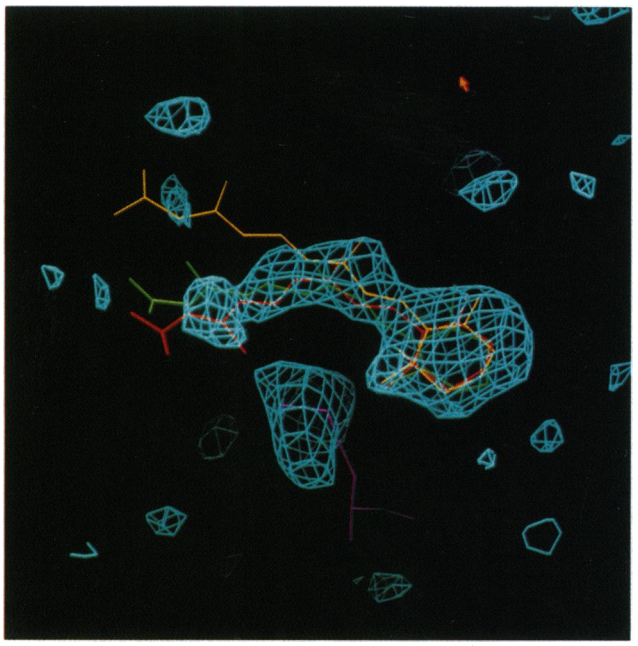

FIG. 2. A 2.4-Å-resolution difference Fourier density. All-*trans*- (yellow), 9-*cis*- (red), and folded all-*trans*- (lime) retinoic acid are fit into the difference electron density. The other large density is due to the side chain of Met-39 (magenta), which has moved into a position different than that it adopts in the apo structure.

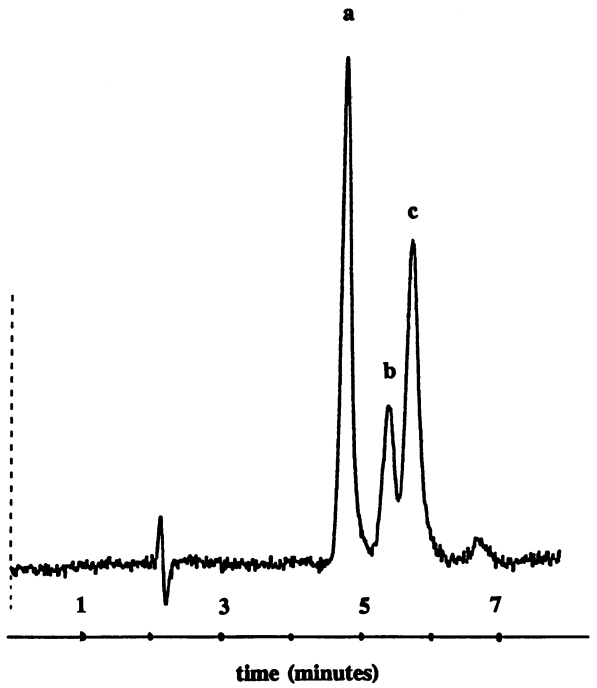

FIG. 3. Chromatogram of the retinoids extracted from holo-crystals of E-RABP. Peaks are observed at retention times indicative of 13-*cis*- (a), 9-*cis*- (b), and all-*trans*- (c) retinoic acid.

lated at this point (Fig. 2) the isoprene tail also became visible. The isoprene tail was clearly not in the extended all-trans conformation seen for that of the retinol in the x-ray structures of the human serum retinol-binding protein and cellular retinol-binding protein types I and II but was "bent" in a horseshoe conformation.

It was not possible to distinguish from the difference electron density whether the retinoic acid was the 9-cis isomer or an all-*trans*-retinoic acid that has been twisted about the C8—C9 bond, as illustrated in Fig. 1*A*. Indeed, the crystal could contain either or both isomers. Although the holoprotein was prepared by the addition of a solution of all-*trans*-retinoic acid to the apoprotein, it was possible that some isomerization occurred to produce 9-*cis*-retinoic acid, which was then preferentially bound by E-RABP. Thus, it was necessary to establish the nature of the bound ligand. Holo-crystals, which were from the same crystallization experiments as that crystal used for the x-ray data collection, were extracted and analyzed for retinoic acid isomers by HPLC (14). The presence of all-*trans*-retinoic acid was confirmed, with some 9-cis and 13-cis isomer also present. The ratio of all-*trans*-retinoic acid to the 9-cis isomer was 2.06/1.0 (Fig. 3). The presence of 13-cis isomer in the crystal extract, despite the fact that it does not compete well with all-*trans*-retinoic acid for binding to E-RABP (12), may be due to the fact that the 13-cis isomer is an abundant product of light isomerization of all-*trans*-retinoic acid (19). The length of time required for the crystallization experiments and the necessary periodic monitoring of the experiments under a light microscope perhaps allowed significant amounts of the 13-cis isomer to accumulate in the crystal.

The demonstration of all-*trans*-retinoic acid in the binding site, confirming previous binding studies (12), then led us to test the ability of E-RABP to bind 9-*cis*-retinoic acid and compare the binding of the 9-cis isomer with that for the all-trans isomer. We incubated E-RABP with mixtures of all-*trans*-, 9-*cis*-, and 13-*cis*-retinoic acid. Protein-bound ligand was separated from free ligand and then extracted for analysis. The relative abundance of the various isomers was quantitated by HPLC (Table 1). The 9-cis and all-trans isomers of retinoic acid bound to E-RABP with almost equal affinity. Affinity of the protein for the 13-cis isomer is significantly lower, at least by a factor of 10, consistent with previous competition studies (12). We also found that TTNPB, a synthetic retinoid that is 80% as active as all-*trans*-retinoic acid in assays of the nuclear receptor RAR-α (3), competed well with 9-*cis*- or all-*trans*-retinoic acid for binding to E-RABP. Although conformationally restricted, rotation about single bonds can position the carboxyl group and the initial ring of TTNPB in the same relative positions found for 9-*cis*- or all-*trans*-retinoic acid in the binding site. That TTNPB does indeed adopt the same horseshoe conformation has been confirmed with x-ray crystallographic studies of TTNPB-soaked crystals of apo-E-RABP (J. Huntington and M.E.N., unpublished observation).

The basic structural framework of E-RABP is an eight-stranded up-and-down β-sheet core that twists into a barrel (Fig. 4). One end of the barrel is closed by amino acid side chains in the barrel interior and amino acid side chains from the amino terminus, which wraps across the back end of the barrel. The front end of the barrel is open and provides entrance to the binding site. The structural motif described

Table 1. Relative abundance of the retinoic acid isomers recovered from E-RABP (C form)

Ligands	Isomer, %		
	13-cis	9-cis	All-trans
All-trans, 9-cis	0	58	42
9-cis, 13-cis	10	90	0
All-trans, 13-cis	6	0	94
All-trans, TTNPB	0	0	58*
9-cis, TTNPB	0	64	0

Data are expressed as percentage of total retinoid recovered for experiments with isomers of retinoic acid. When TTNPB was present, recovery of retinoic acid isomers is expressed as percentage recovered compared with recovery without TTNPB; TTNPB was not quantitated.
*Ligands were provided in equimolar amounts.

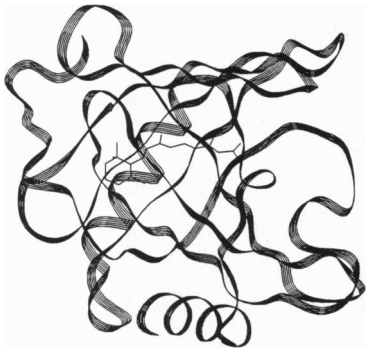

FIG. 4. Ribbon diagram of the C_α backbone of E-RABP with retinoic acid in the binding cavity.

for E-RABP was first described for the human serum retinol-binding protein, a protein with which E-RABP shares slightly <20% amino acid sequence identity (20). A detailed comparison of the two proteins will be presented with the report of the apoprotein structure (16).

In the inside of the β-barrel is a large and deep cavity that forms the retinoic acid-binding site and binding-site entrance. The deepest part of the cavity is lined with hydrophobic amino acids. Charged amino acids are found in the cavity proximal to the binding-site entrance. The entrance itself, formed by the inside faces of the two β-sheets, has both hydrophobic and charged amino acids. The side chains that line the binding cavity are Phe-6, Phe-11, Trp-15, Met-39, Val-41, Leu-48, Leu-50, Phe-76, Val-78, Arg-80, Lys-85, Val-87, Val-89, Ala-98, Ile-100, Ile-102, Lys-115, and Tyr-117. The five aromatic amino acids, Phe-6, Phe-11, Trp-15, Phe-76, and Tyr-117, are deepest in the binding cavity. They form a semi-circle at the back end of the barrel, and the β-ionone ring of the retinoic acid is at the center of this semicircle. More proximal to the entrance are Arg-80, Lys-85, and Lys-115. The deepest of these residues is Lys-115, the α-carbon of which is located in the hydrophobic portion of the binding site. The side chain of Lys-115 stretches out along the "bottom" side of cavity toward the binding-site entrance so that the positively charged amino group is at the entrance end of the binding site, positioned to hydrogen bond with the ligand carboxylate. Lys-85 is at the interface of the large entrance to the site and the binding cavity itself and comes from the "top" side of the cavity to hydrogen bond with the retinoic acid carboxylate. The binding-site entrance is lined with both hydrophobic and charged amino acids. His-111 and Arg-80 come from the inside face of the "top" β-sheet, and Glu-17 and Glu-63 come from the inside face of the "bottom" β-sheet. If one tries to model 13-*cis*-retinoic acid in the binding site, the carboxylate of the ligand is positioned unfavorably close to Glu-17. This observation would explain the reduced affinity of E-RABP for the 13-cis isomer.

The retinoic acid fits snugly into the hydrophobic pocket of E-RABP, as the contours of the protein are complementary to those of the hormone. The surface area of the bound retinoic acid accessible to a solvent molecule is <30 Å^2, compared to an area of 660 Å^2 for the free ligand. The solvated portion of the retinoic acid corresponds to the carboxylate region. To illustrate complementarity of the fit of the ligand in the protein-binding site we have calculated the accessible surface area of the holoprotein without ligand and the accessible surface areas of 9-*cis* and all-*trans* (in the conformer observed in the protein-binding site)-retinoic acid. In Fig. 5 we have taken cross sections of the surface representation of E-RABP, so that the internal contours of the binding site are visible. In Fig. 5*A*, calculated for holoprotein without ligand, one can see the deep binding cavity. For Fig. 5 *B* and *C* we have fit the surface representations of

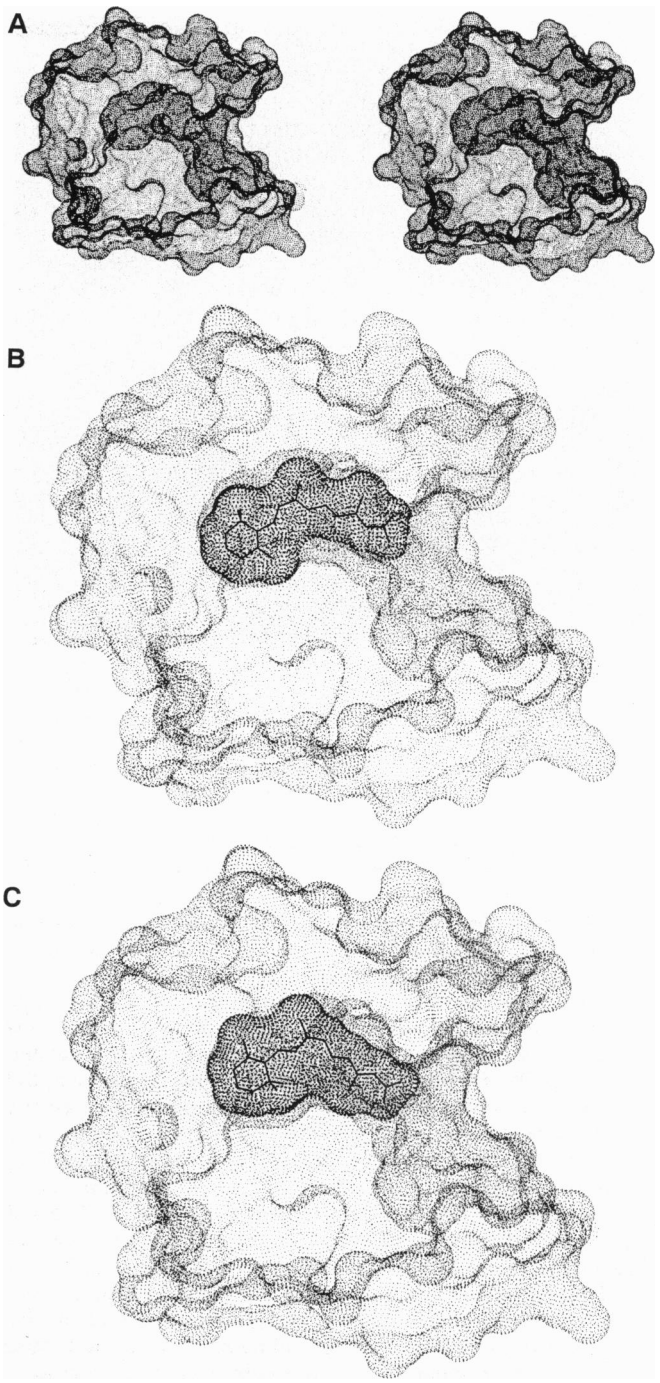

FIG. 5. Cross sections of the surface representations of E-RABP in which the binding cavity is visible. Dots mark the surfaces accessible to solvent molecules. The figures were generated with Biosym software that incorporates the algorithm of Connolly (21). A solvent probe radius of 1.6 Å was used with the coordinates for all nonhydrogen atoms. (*A*) Holo-E-RABP without retinoic acid (stereo). (*B*) Holo-E-RABP as in *A* but with the surface of all-*trans*-retinoic acid mapped in darker dots. (*C*) Holo-E-RABP as in *A* but with the surface of 9-*cis*-retinoic acid mapped in darker dots.

all-*trans*- and 9-*cis*-retinoic acid, respectively, into the binding cavity of the holoprotein (as shown in Fig. 5*A*). These latter two representations, in addition to illustrating the complementarity of the surfaces of binding cavity and ligand, suggest that the binding site of E-RABP may be able to recognize both 9-cis and all-trans isomers of retinoic acid because the site can accommodate the isoprene methyl groups in two different conformations.

DISCUSSION

The isomer specificity of E-RABP that was predicted by the shape of the electron density of the ligand in an x-ray crystallographic study was confirmed experimentally. The "folding" of the all-*trans*-retinoic acid isoprene tail suggests a mechanism by which the same protein-binding site can recognize both all-*trans*- and 9-*cis*-retinoic acid while maintaining many of the same protein–ligand contacts. A folded all-*trans* and 9-*cis*-retinoic acid can be superimposed so that the shapes of the volumes occupied by the two structures are roughly the same, as illustrated in Fig. 5 *B* and *C*. To make the all-*trans*-retinoic acid most resemble the 9-cis isomer a rotation about C6—C7 and C8—C9 is necessary. In the variety of crystal structures of retinoids available, the β-ionone ring has been seen to be in either of these conformations (e.g., ref. 11). In the various crystallographic structures of all-*trans*-retinoids reported, the isoprene tail is invariably in the extended conformation. In addition, in the three structures of retinol-binding proteins determined, the isoprene tail was found in the extended conformation. These x-ray structures include the human serum retinol-binding protein, which has the same structural motif as E-RABP. In contrast to E-RABP, human serum retinol-binding protein does not discriminate between retinol and retinoic acid. The ligand binding site of this retinol-binding protein resembles that for E-RABP in that the retinol fits snugly in the binding cavity; however, in E-RABP the retinoic acid is placed much deeper into the β-barrel, and the ligand carboxylate interacts with amino acid side chains on the inside of the β-barrel. The retinol-binding sites of the intracellular retinol-binding proteins CRBP and CRBPII are also sandwiched between two layers of β-sheets. However, in these proteins, which have their own common structural motif, the tail end, rather than the β-ionone ring, is deepest in the barrel, and the internal binding cavity does not conform extensively to the shape of the ligand due to the presence of multiple water molecules in the binding cavity.

We see here that upon binding to E-RABP, retinoic acid adopts a conformation in which the tail is now "folded" instead of extended. For the retinoid to assume this conformation, a transitory break in the conjugation across the length of the isoprene tail must occur, but once the isoprene tail adopts a "pseudo-cis" conformation, conjugation is restored. In the binding of retinoic acid to E-RABP there is also a conformational change in the protein. The side chain of Met-39 must move to allow the β-ionone ring to enter the innermost region of the deep and hydrophobic binding pocket. The mechanism described here for retinoid recognition, which requires that the retinoid undergo a conformational change, could be the same type of mechanism utilized by RAR-α so that it can respond to both the 9-cis and all-trans isomers of the hormone. It remains to be confirmed by further crystallographic studies with the variety of retinoids that activate RAR-α whether the binding site of E-RABP can serve as a structural model for the receptor-recognition site. Structural homology of catalytic sites has often been observed in proteins with no overall structural homology. The pocket is primarily hydrophobic in nature and sequence identity between E-RABP and RAR is not a necessary prerequisite for binding-site structural homology: a hydrophobic pocket with similar contours could be created by an assortment of hydrophobic side chains in the context of other structural motifs.

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